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Improved Detection Sensitivity of a Biological Simulant: Delivery of Chemical Labels to the Cell Surface by Adsorption of Polyethyleneimine Derivatives

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Improved Detection Sensitivity of a Biological Simulant: Delivery of Chemical Labels to the Cell Surface by Adsorption of Polyethyleneimine Derivatives

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Abstract: The use of biological simulants in testing detection/identification instruments often employs antibodies to quantify the amount of simulant or sometimes as an integral part of the detection suite. At present, the bacterium *Pantoea agglomerans* (formerly *Erwinia herbicola*, *Eh*) is an approved vegetative agent simulant, however, *Eh* detection sensitivity is disappointing in standard methods such as ELISA, using various anti-*Eh* antibody preparations, including antibodies that have been raised against surface-modified *Eh* in attempts to increase *Eh* immunogenicity. In addition, anti-*Eh* antibodies exhibit low specificity for *Eh*, and react almost as strongly with other enterobacterial species, e.g., *E. coli*, limiting their value for identification purposes. Therefore, we are pursuing two alternative strategies, genetic manipulation of *Eh* cell surface components, and chemical modification of the negatively charged surface of *Eh* to introduce specific groups for detection with antibodies and/or with other highly specific and sensitive tests already available. Here we report progress on the development of a method to deliver onto the surface of *Eh* large numbers of small molecule labels multiply attached to a polycationic polyethyleneimine (PEI) carrier by strong electrostatic adsorption of the derivatized polymers to cells. Biotin (Bio) or dinitrophenyl (DNP) groups were covalently linked to PEI of average MW 60,000, bearing approx. 1,400 positive charges, by reaction of 1% w/v PEI with *N*-hydroxysuccinimide-biotin (NHS-biotin) or dinitrobenzenesulfonic acid (DNBS), respectively. Limiting amounts of NHS-biotin and DNBS reagents were employed to incorporate either ~ 50 or ~ 100 Bio or DNP groups per molecule of PEI, leaving a majority of positively charged groups unaltered. After overnight incubation with PEI derivatives and extensive washing to remove unadsorbed polymer, assays using anti-DNP antibodies and streptavidin-peroxidase showed high levels of the PEI derivatives associated with *Eh* cells. In incubations with 0.1, 0.2 and 0.9% w/v PEI polymer containing on average 52 biotin groups per molecule (PEI-Bio 52), approximately 14,000, 29,000 and 50,000 molecules of PEI-Bio 52 were bound per cell, respectively. Dot blots assays using streptavidin-peroxidase with enhanced chemiluminescence detection showed much higher detection sensitivity compared with anti-*Eh* antibodies used in standard colorimetric ELISA assays. In addition to high sensitivity and specificity, cell surface adsorption of PEI derivatives provided a simple and versatile method in which much higher cell viability was maintained compared with procedures using direct chemical labeling of cells with low molecular weight reagents.

Introduction

Erwinia herbicola is a rod-shaped, non-pathogenic, Gram-negative facultative anaerobe, belonging to the family Enterobacteriaceae. As such, this organism is an appropriate simulant for *Yersinia pestis* and other Gram-negative pathogens (e.g., *Francisella tularensis*). *E. herbicola* has been approved for environmental release at Dugway Proving Ground, and at present is the common simulant in tests requiring vegetative bacteria. (It is important to note that the accepted scientific name of the strain used has been changed to *Pantoea agglomerans* ATCC 33243, however we will use *Erwinia* or *Eh* synonymously here, as the strain is commonly termed this way at ECBC and this designation has been used in reports.) *Eh* according to the testers of equipment is presently deficient in two ways. The first is that its antigenicity is not adequate as a backup for other identification procedures being used. The second is that the survivability of *Eh* is quite low when it is aerosolized to mimic an aerosol threat (1,2). The aspect of survivability is currently under investigation by others at the Aerosol Science group at the ECBC.

The present study follows from our earlier report (3) on efforts to obtain improved antibody-antigen responses to facilitate advances in the use of *Eh* as a simulant. In those studies, antibodies were raised in rabbits against native *Eh* and against preparations of *Eh* chemically modified by reaction with dinitrobenzensulfonic acid [introducing numerous dinrophenyl (DNP) groups onto cell surface components]. DNP-labeled *Eh* was readily detected with high sensitivity using anti-DNP antibody preparations [using commercial anti human serum albumin-DNP (anti HSA-DNP) antibodies as well as our anti *Eh*-DNP antibody preparations]. However, all of the *Eh* cells were killed in the chemical labelling procedure, and could not be further monitored by microbiological culture methods that require viable cells. Furthermore, the anti *Eh*-DNP antibodies were at best only marginally better at detecting native *Eh* compared with existing *Eh* antibodies. In addition, all antibody preparations studied so far suffer from another problem -- that of cross reactivity with other enterobacterial species, e.g., *E. coli*, which makes it difficult or impossible to use them for specific identification of *Eh*.

The focus of the present work is on the development of alternative, milder methods for delivering large numbers of chemical groups to the surface of *Eh*. (A separate strategy using molecular genetic techniques to introduce specific peptide tags on the surface of *Eh*, is also being pursued, and some recent advances along those lines are reported here, as well.) Herein we describe an efficient method to produce biotin and DNP derivatives of a high molecular weight cationic polymer, polyethyleneimine, which can be readily adsorbed to the surface of *Eh* under mild conditions. Cells exposed to PEI multiply labeled with biotin or DNP groups were detectable with high sensitivity using commercially available reagents (streptavidin conjugates or anti-DNP antibodies, respectively), and substantial viability of the cells was maintained. The use PEI as an inexpensive vehicle for the delivery of multiple small molecule groups provides a simple and versatile method that, in principle, also could be used to modify *Eh* or other potential vegatative simulants with a wide range of other chemical species including synthetic peptide antigens relevant to agent surface markers.

Results and Discussion

Chemical modification strategy

The Gram-negative enterobacterial cell envelope, shown schematically in Fig. 1, contains many different complex lipid, carbohydrate and protein components that can interact with the external environment, and thus are of potential use for the development of detection strategies (4). We first tested rabbit anti-*Eh* antibodies available from the ECBC Critical Reagents Repository (CRP) by Western blot analyses, and showed that these antibodies react with a number of such components, both proteins and polysaccharides. Moreover, and perhaps not surprisingly, the available antibodies showed marked cross reactivity with similar components on other bacteria such as *E. coli* (3). In that study, we also compared the detection sensitivity using antibodies raised against preparations of *Eh* that had been labeled with dinitrophenyl groups on the rationale that introduction of chemical groups in the vicinity of a weakly antigenic structure results in antibody production not only against the newly attached chemical group, but also often elicits a stronger immunological response against the native structure as well. This property was previously pointed out to one of us (B.V.B.) at an earlier meeting by Dr. Jose-Luis Sagripante, see also (5).

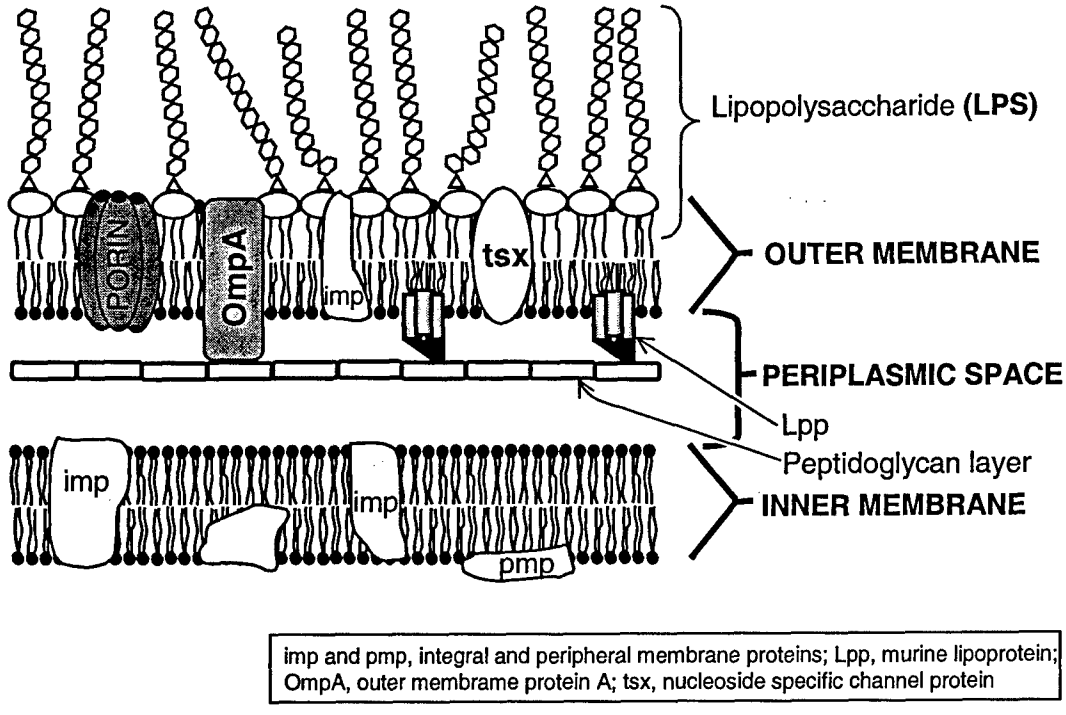


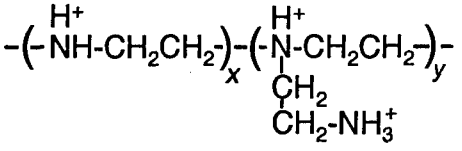
Figure 1. Structure of the Gram-negative enterobacterial cell envelope (4).

Although only a marginal increase in detection sensitivity of the native structures was observed, affinity purified antibody preparations provided a 4-fold increase in detection sensitivity. Moreover, in the course of preparing and testing such preparations, we discovered that a specific sub-population of the original antibodies was enriched – those reacting against lipopolysaccharides – while a number of cell protein components

were not well recognized by the affinity purified antibodies, suggesting that the most highly immunogenic epitopes of these proteins are not accessible on the cell surface under native conditions, and may reside inside the membrane spanning regions or within the periplasm. Our procedure to introduce DNP groups used a reagent that reacts with amino groups, which are present in outer membrane proteins, but are generally not found in extracellular polysaccharide structures. Thus, the results prompted us focus on developing an alternative strategy using different reagents to carry out DNP modification of the extracellular polysaccharides. The method described here takes advantage of the fact that natural and synthetic polycationic polymers adsorb strongly to cells due in large part to the large number of negative charges on the cell surface (e.g., from acidic groups that are present in the core structure of each LPS molecule and on each trisaccharide repeat unit making up polysaccharide chains of enterobacterial common antigen, ECA) (4,6).

Preparation of PEI vehicles bearing multiple labels (PEI-biotin and PEI-DNP)

Polyethyleneimine (PEI), which is an inexpensive, commercially available polymer of ethyleneimine, bears multiple pimary, secondary and tertiary amino groups that are positively charged at neutral pH, with the general structure shown below.



We reacted PEI with a molecular mass of approximately 60,000, containing an average of ~ 1400 ethyleneimine groups per molecule, with limiting amounts of amino-reactive chemical reagents to synthesize two different kinds of derivatives of PEI with varying degrees of incorporation of DNP and biotin groups, ranging from approximately 25 to 150 such groups per PEI molecule, while leaving the majority of amino groups unmodified. Schematically, the products, designated PEI-DNP and PEI-biotin are represented as shown in Fig. 2. Before incubating with cells, all PEI and PEI derivatives were extensively dialyzed against phosphate buffered saline (PBS) to remove any toxic unreacted reagents and low molecular weight products and to restore neutral pH.

DNP derivatives of PEI were prepared by reaction of 1% w/v PEI (avg. MW 60,000) with 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.1 M potassium hydrogen carbonate (0.05 M $KHCO_3$/0.05 M K_2CO_3), pH 10.4, at room temperature for 24 hours. Different concentrations of DNBS were employed, with 5 mM, 10 mM and 20 mM yielding soluble PEI-DNP derivatives bearing an average estimated number of DNP groups per PEI molecule of 25, 50, and 100, respectively (products designated as $PEI\text{-}DNP_{25}$, $PEI\text{-}DNP_{50}$ and $PEI\text{-}DNP_{100}$, respectively). DNP group incorporation was monitored spectrophotometrically using a molar absorptivity coefficient of 14.7 mM^{-1} at 360 nm. Biotinylation of PEI using biotin *N*-hydroxysuccinimide (Bio-NHS) at pH 8.3 was carried out using 1% PEI solutions prepared by first dissolving PEI in 0.05 M $NaCO_3$ and then adjusting the pH to 8.3 with 1M HCl. Bio-NHS is commercially available, but it was substantially less expensive for us to prepare large amounts of Bio-NHS in a

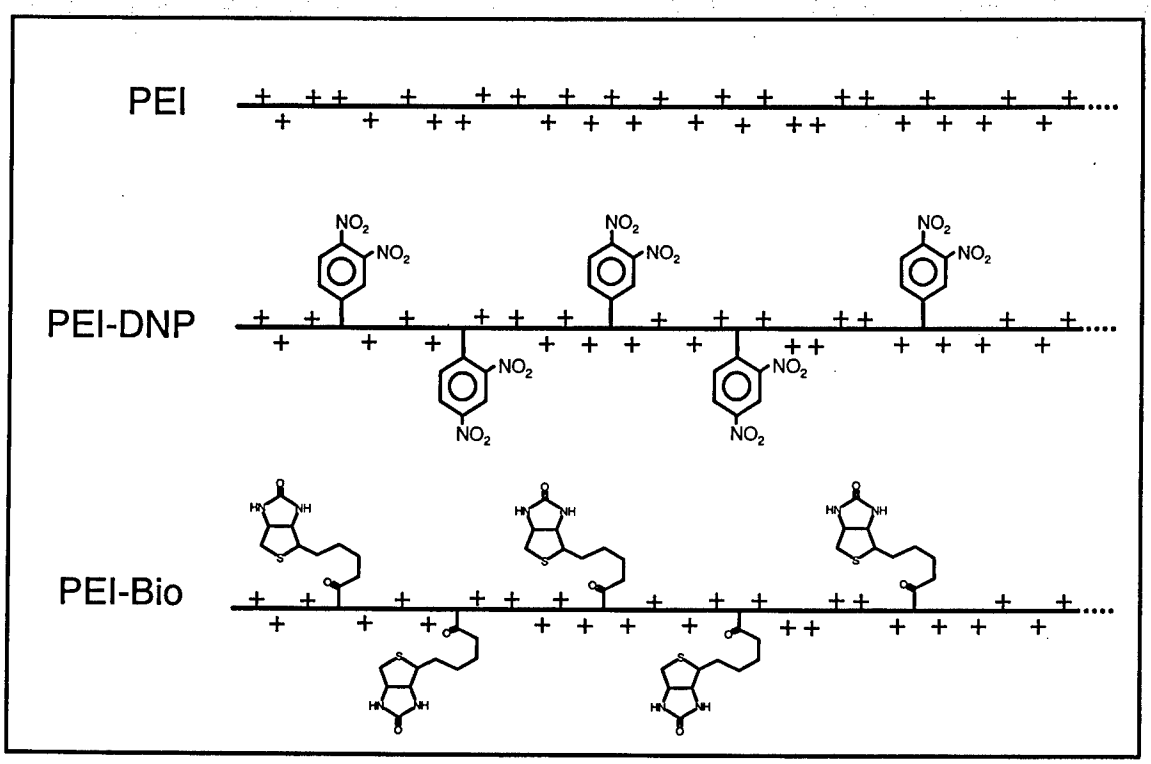


Figure 2. Schematic representation of PEI derivatives. (see text for synthetic methods used)

reagent form that can be used directly in reactions with PEI. Biotin *N*-hydroxy-succinimide reagent in dimethylformamide (DMF) was prepared by the method of carbodiimide coupling, and was carried out by addition of 2.5 g *N*-ethyl-*N*'-(3-dimethyl-aminopropyl)carbodiimide (EDC) to a solution containing 2.44 g biotin and 1.5 g *N*-hydroxysuccinimide dissolved in 130 ml of dry DMF with stirring overnight. The reagent is stable and can be for several months when kept at –20 °C. The number of biotin groups incorporated per molecule of PEI was varried by reaction with different amounts of Bio-NHS reagent. PEI bearing an average of 26 and 52 biotin groups was prepared by adding one volume of Bio-NHS reagent to 18 or 9 volumes of 1% PEI bicarbonate solutions, pH 8.3, respectively. Addition of 1 volume of Bio-NHS reagent to 18 volumes of 0.5% PEI bicarbonate solution was used for attachment of around 104 biotin groups per molecule PEI. (The final PEI-biotin preparations are designated PEI-Bio_{26}, PEI-Bio_{52}, and PEI-Bio_{104}.) Under the reaction conditions, hydrolysis of Bio-NHS also takes place, however, control reactions in which the release of NHS was monitored spectrophotometrically at 260 nm (in the presence and absence of PEI) indicated that transfer biotin to PEI is a much faster process. All PEI-biotin and PEI-DNP products were extensively dialyzed before incubation with *Eh* cells.

Incubation of PEI and derivatives with Eh

As shown in Fig. 3, incubation of *Eh* cell suspensions with low concentrations of PEI (as well as with PEI derivatives, not shown) causes aggregation of *Eh* cells, but cells exposed to higher concentrations of PEI remain suspended. A similar effect has been noted for *E. coli* in the presence of cationic diethylaminoethylacrylate polymers (6) and reflects principles of colloid behavior where it is known that high molecular weight substances cause the destabilization of dispersions, whereas in high concentrations act as stabilizers.

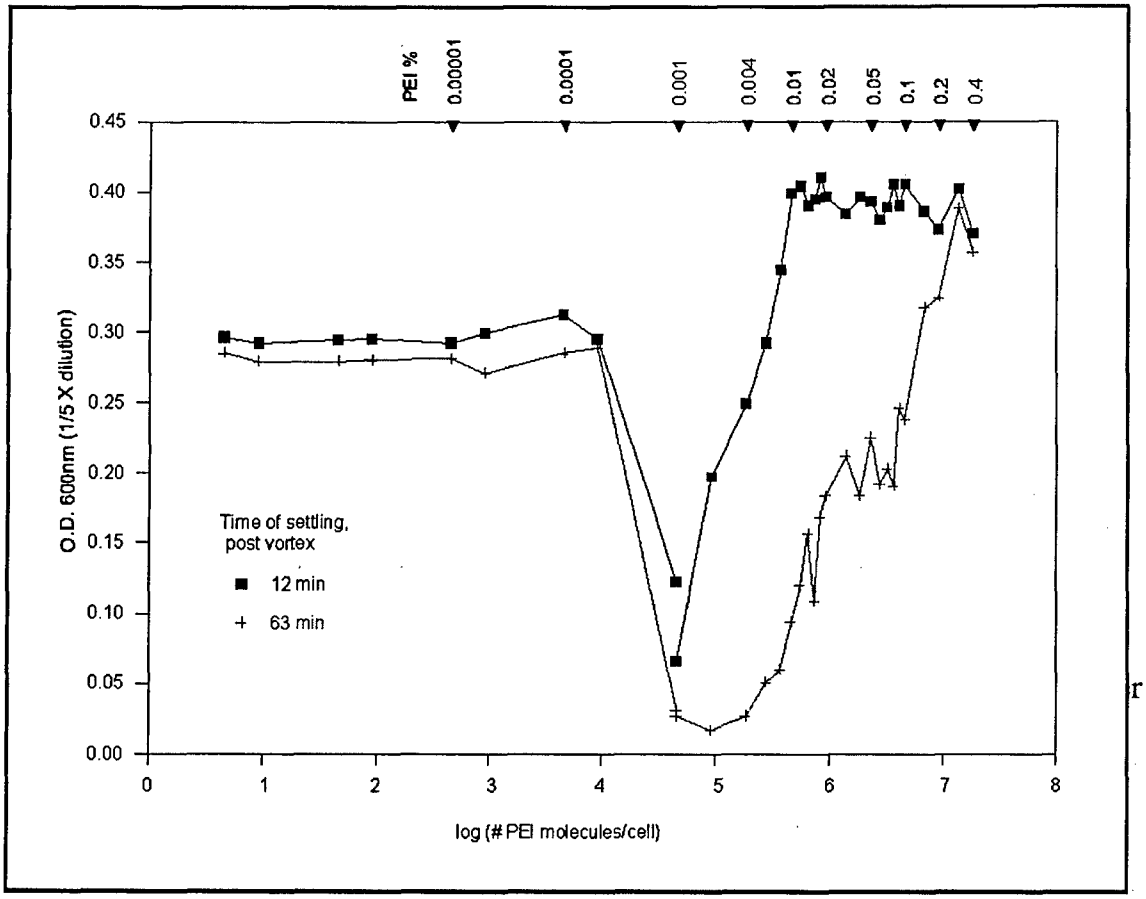


Figure 3. Flocculation phenomenon encountered with *Eh* incubated with different concentrations of PEI.

The number of cells remaining viable following incubation with PEI and PEI derivatives was also measured, as shown in Fig. 4.

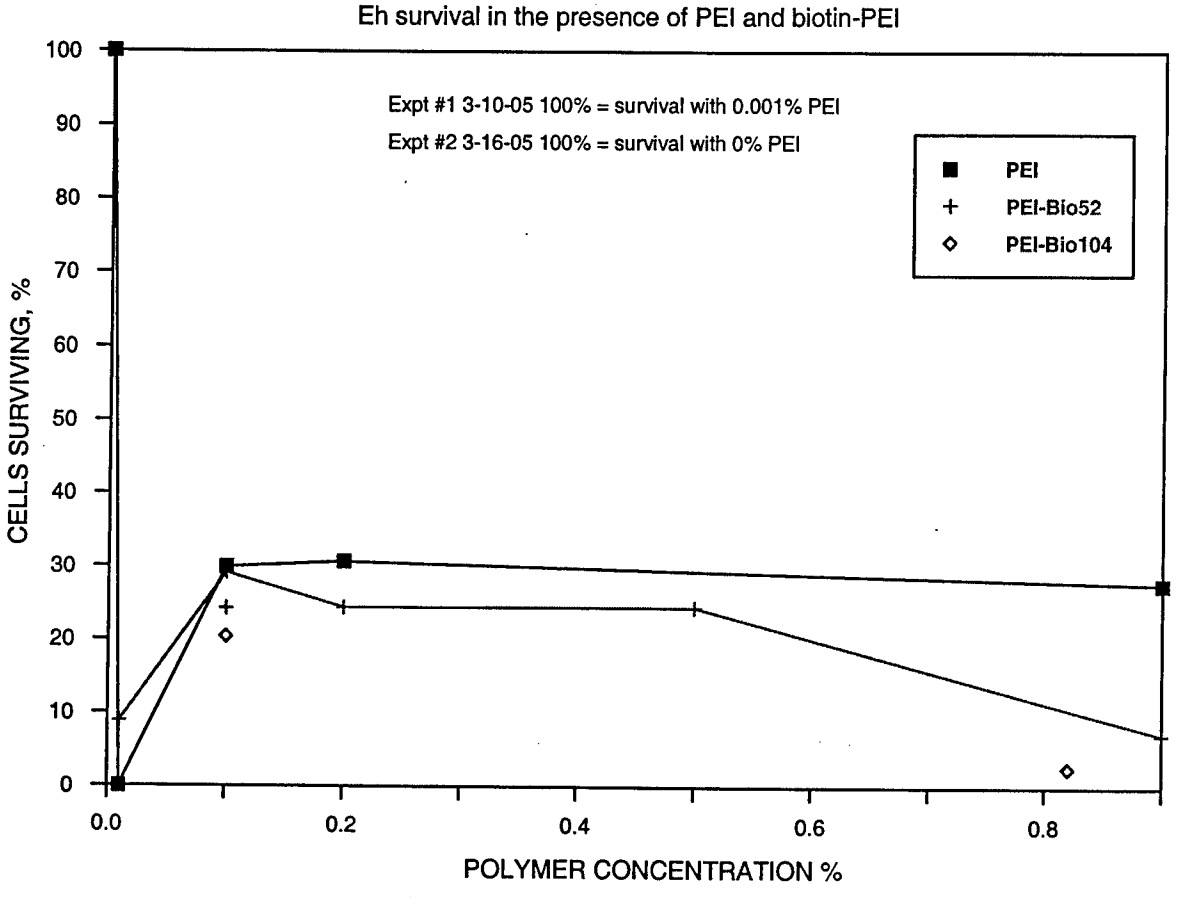


Figure 4. Viability of *Eh* incubated overnight with different concentrations of PEI and PEI-biotin derivatives. Plate counts were used to determine cell survival after overnight incubation of cells (2 x 10^9 cells/ml) with the indicated concentrations of PEI and PEI derivatives.

Here it can be seen that large numbers of viable colonies are easily obtained at concentrations of 0.1 % PEI and above, but that flocculation at low concentrations is associated with low recovery of viable colonies. Whether or not this means that cell killing is actually taking place at the low concentrations remains to be established, since flocculation may interefere with the physical ability to distribute single cells in the process of plating without actually decreasing the number of viable cells. A effective method for disaggregation would be helpful to establish this.

Detection of Eh by sensitive specific assays for labeled PEI derivatives adsorbed to the surface of Eh cells

The rationale for detection of cells labeled by PEI derivatives is based on the finding that PEI is strongly adsorbed to cells, and remains associated even after extensive washing by several repeated centrifugation and resuspension steps. High detection sensitivity is expected since each molecule of PEI carries multiple labels, thereby enhance signal

strength. In addition, survivability is maintained when PEI or its derivatives are adsorbed to the cells, presumably since unlike low molecular weight reagents the very high molecular of PEI does not allow it to enter into the cell where toxic effects on metabolic process may occurr. Further, adsorptive delivery of labels to the cell surface on a polymeric vehicle (attached to the polymer in a previous chemical step under harsher conditions) can be carried out at neutral pH 7 in the absence of all other low molecular weight reagents, whereas it is more often difficult or possible to carry out direct covalent chemical linkage to cell surface components under such mild conditons. The results of a sensitive assay for biotin using streptavidin-peroxidase conjugates to detect *Eh* cells labelled with PEI-biotin are shown in Fig. 5.

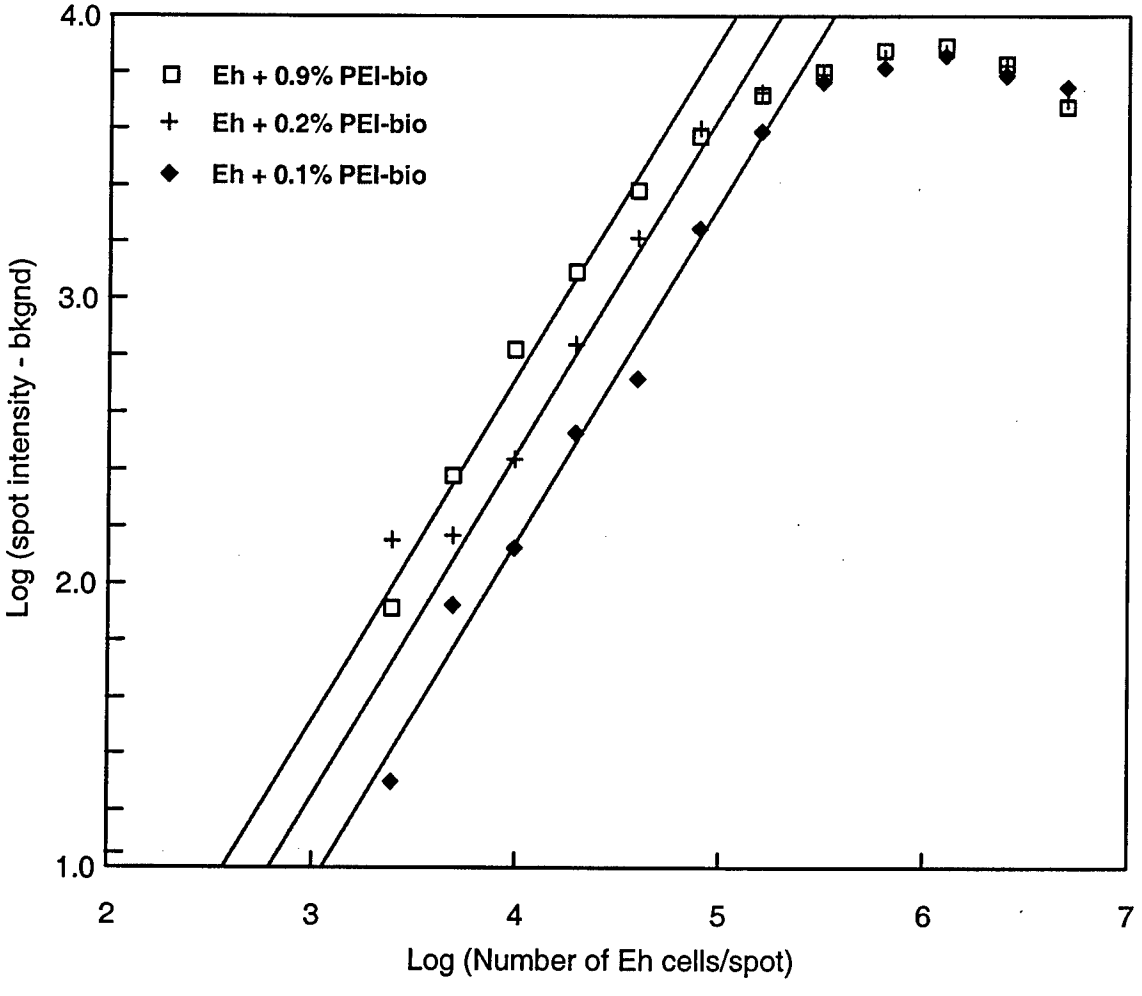


Figure 5. High sensitivity dot blot analysis of *Eh* cells exposed to different levels of PEI-biotin 52.

Eh cells (2 x 10^9 cells/ml) were incubated overnight in PBS containing three different concentrations of PEI-Bio_{52} indicated (0.1, 0.2 and 0.9% w/v). Then three washes were performed with PBS, and the cells were finally resuspended in PBS with 10% glycerol (in 1/5 th the original volume to give 1 x 10^{10} cells/ml). A 100-fold dilution was performed by adding 100 μl of the cells to 9.9 ml of PBS, and 50 μl of this was applied to

the first spot in a series (i.e., 5 x 10^6 cells on the first spot, plotted data points furthest to the right). A series of eleven 2-fold dilutions were performed starting from the 100-fold diluted material, and 50 μl from each was applied to the remaining spots. A Hybond N nylon membrane was pretreated with 1% polyacrylic acid and after spotting the labeled cells, blocking was performed for 50 min in a solution of PBS containing 0.01 % bovine serum albumin. The blot was then washed four times in PBS containing 0.05% Tween 20 (PBS-T), incubated with Streptavidin-peroxidase in PBS-T for 1 h, and washed five times in PBS-T. Chemiluminescent detection was carried out using the ECL Western blotting analysis system reagents, PRN 2109 from Amersham Biosciences with Kodak X-OMAT AP film. Densitometry was carried out on 50 mm diameter circular areas of interest, with spot intensity determined as integrated absorbance. The background spot intensity value was approximately 100 (arbitrary absorbance x area units).

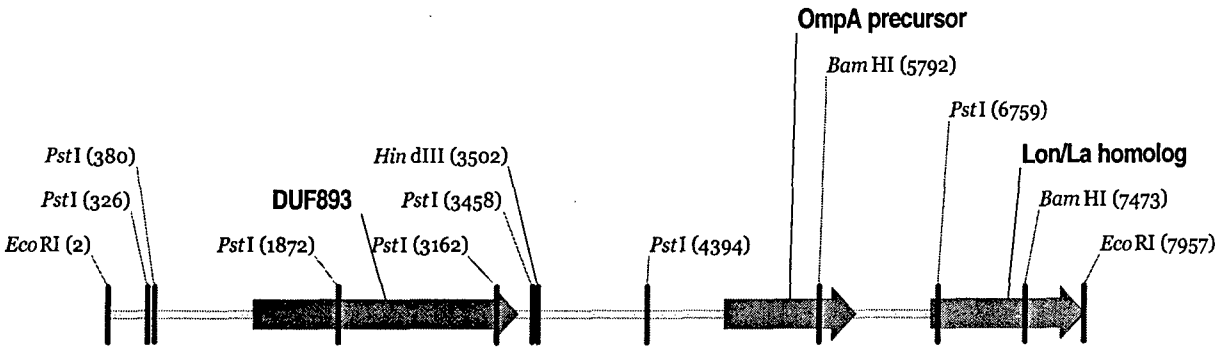
The experiment also included analysis of standard PEI-biotin 52, not shown, to establish the signal intensity produced by known amounts of the modified polymer. From this, it was calculated that approximately 14,000, 29,000 and 50,000 molecules of PEI-Bio_{52} were bound per cell in the incubations with 0.1, 0.2 and 0.9% w/v PEI polymer, respectively. In addition, the amount of PEI adsorbed to cells appears to be proportional to the concentration of PEI derivative in the incubation mix in the 0.1 to 0.2% PEI range, but falls off above that. The detection limit was around 8,000, 4,000 and 2,000 cells in the respective incubations – reading horizontally across at around a value of 2 on the log(spot intensity-bkgnd) axis, i.e., at about 100 counts above background (the value where S/N . 1). The log-log plot under idealized conditions (signal intensity strictly proportional to analyte concentration) should have a slope of 1. The lines drawn in the Fig. 5 have a slope of 1.2, which is not too unreasonable for a method involving heterogeneous reaction chemistry taking place on both solid and liquid phases.

DNP-PEI was simularly found to be relatively non-toxic to cells. Preliminary experiments indicate that substantially larger amounts of DNP-PEI is adsorbed compared with PEI-biotin – on the order of approximately 500,000 PEI-DNP_{50} molecules per cell, or roughly ten times more than DNP-biotin. Detection of viable *Eh* labeled with DNP-PEI in an ELISA format was readilly accomplished using commercially available anti DNP-HSA antibodies (data not shown). Labeling of *E. coli* with PEI-DNP also was found, but somewhat lower levels of DNP incorporation were seen, approximately 20% of that relative to *Eh*. Nonetheless, detection of PEI-DNP labelled *E. coli* was accomplished with good sensitivity, and the results establish that use of PEI derivatives is also applicable for labelling cells other than *Eh*.

Genetic modification of Eh — preliminary results

In addition to developing chemical labeling strategies for detection of *Eh*, it would be useful to have effective methods to carry out genetic manipulation of the organism, and potentially generate modified strains displaying specific molecular determinants to aid in the identification and use as improved agent simulants. Therefore, we have been interested in modifications relating to a major outer membrane protein (designated OmpA) present in high concentrations on the *Eh* surface. Therefore, we cloned and sequenced an ~ 8 kb EcoRI fragment of the *Eh* genome which contains OmpA, two

additional genes (DUF893 and Lon/La homolog) and significant intergenic stretches, as shown in Fig 6. We have shown that OmpA is readily detected by Western blot analysis using anti-*Eh* antibody preparations, and can be clearly distinguished from *E. coli* OmpA following SDS gel electrophoresis on the basis of its slightly larger in molecular mass. We constructed an expression vector containing the *Eh ompA* gene under the control of the *lac* promoter, and have characterized the heterologous expression of *Eh* OmpA in *E.coli*. The results showed that a significant amount of *Eh* OmpA undergoes normal proteolytic processing, i.e., removal of the first 21 amino acids at the N-terminus, as



needed for insertion and crossing of the inner membrane prior to arrival at the outer

Figure 6. *Erwinia herbicola* 8 kb genomic fragment containing the complete *Eh* OmpA gene with signal sequence needed for membrane transport and upstream regulatory regions. Sequence information has been obtained for the entire 7961 bp region, but has not yet been submitted for publication in the public database.

membrane. Thus, it is reasonable to assume that following heterologous expression in *E. coli*, significant amounts of *Eh* OmpA end up properly positioned in the outer membrane.

Having characterized the heterologous expression of *Eh* OmpA in *E. coli*, we are now in position to express genetically modified *Eh* OmpA in *Eh* itself, as a means for modification of *Eh* surface epitopes to allow for specific and sensitive detection. Studies are underway to establish whether homologous recombination can be employed, e.g., to insert defined constructs into specific regions of the 8 kb *Eh* genomic segment, thus improving the genetic versatility *Eh*.

Summary

Polymeric PEI derivatives were effective at delivering multiple biotin and DNP groups to the surface of *Eh* cells, allowing for highly sensitive, direct detection of cells so-labelled. A substantial degree of cell viability was maintained. Although, PEI-induced flocculation was observed at lower concentrations of PEI, high concentrations in the range of 0.1 to 0.5% were optimal to provide high levels of label incorporation, maintain cell viability, and cause minimal cell aggregation. *E.coli* cells also were readilly labeled with DNP-PEI derivatives indicating that the method has the potential to be applied to other organisms that may be of possible future interest as vegatative simulants besides *Eh.*

Cloning and sequencing of an 8 kb genomic fragment containing the complete *Eh* OmpA gene and other information of interest was accomplished, which is expected to be a benefit for future studies on genetic systems in *Eh.*

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